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Biophysics of Bacterial Walls Viewed as Stress-Bearing Fabric

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INTRODUCTION

D'Arcy Wentworth Thompson's book (101) was seminal in pointing out the key role of physical and engineering concepts in biology. Papers by Mitchell and Moyle (79) and Weidel and Pelzer (113) were key in introducing the same approach to microbiology. The former workers showed that bacterial cells had a high osmotic pressure. The latter workers explained how it could be contained, proposing that the peptidoglycan layer formed a covalently closed "sacculus." It was a logical extension of these ideas that led to the surface stress theory (10, 38, 42–46, 50–60, 85). This theory outlined the strategies that different microbial cells have evolved to permit them to grow in spite of their containment inside a constraining, stretched network. All of the strategies depend on adding and linking nascent oligopeptidoglycan to the stress-bearing wall before critical cleavage events take place that permit growth and division of the procaryotes, which have no protein such as actin to force mechanical movement. In this way the wall remains strong during the enlargement process. The details are somewhat different for the sidewalls and poles of gram-positive organisms and wholly different for the sidewalls and poles of gram-negative rods. Probably the gram-positive and -negative cocci grow by using the developmental strategies used for the poles of these two basic wall types.

Recently, new approaches have been developed to measure the turgor pressure in bacteria (59, 85) and the stretch of the sacculus surrounding the living cells (57). It is, therefore, appropriate to bring together microbiological facts with old and new knowledge of the properties of fabrics and other materials, both man-made and natural, to try to understand the mechanism of the sacculus formation and function in the living bacterial cell.

STRESS-BEARING PART OF THE WALL: A WOVEN OR NONWOVEN CLOTH?

Ordinarily we think of fabrics as being knitted or woven from thread in a regular, systematic way. However, the alternative type of fabric is the "nonwoven" type and is much less demanding in its mode of construction (22, 29, 30, 69). For both types, the thread may be composed of a single monofilament or spun from many shorter filaments. The thread may be inherently rodlike and fairly inflexible, or it may be coiled in various ways. Fabrics may have appendages like the nap of a rug. Perhaps there are analogs of these features in the bacterial cell wall structure. Certainly an analogous feature in the bacterial wall would be the nonstress-bearing material such as teichoic acid or the partially autolyzed wall of gram-positive organisms that appears as a fringe of material in many electron micrographs.

The original concept of Weidel and Pelzer (113) corresponds to a fish net where the cords are knotted at regular intervals. In the murein, these are at the intersections of the peptide chains with the saccharide chains. Thus, the muramic acid moieties serve the role of the knot in that they allow three chains to be covalently attached. The evidence accumulated since their work is entirely consistent with this point of view. They also theorized that the fabric is a regular network covering the cylinder of the cell in such a way that chains of one kind went entirely around the cell and formed a closed circle. Most workers believe they were wrong in this assumption. However, such a model provides a logical

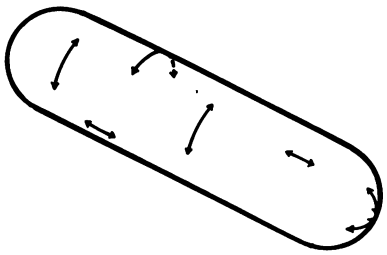

FIG. 1. Tensile stresses in the surface of a rod-shaped organism. The length of the two-headed arrows are proportional to the stresses in the plane of the wall. Because the wall is thin compared with each of the other dimensions, the wall must move until there are no resultant forces normal to the surface. Note that the stress is twice as great in the hoop as in the meridional direction on the cylindrical surface.

explanation of how the bacilli can be rod shaped instead of spherical. The recent models of Mendelson and co-workers (74–78, 105) and of Burman and Park (13) are related to the original model of Weidel and Pelzer (113). The new variations propose that the oligosaccharide chains form a helix around the cylinder of the rod-shaped organism and have a continuity in form, if not in chemical structure, several or many times the circumference of the rod. This class of models views the stress-bearing portion of the cell wall as a "woven" fabric in the lexicon of the textile engineer.

The alternative view is that the wall is formed as a more or less randomly oriented network of short oligoglycans, cross-linked by chance interaction with membrane-bound enzymes. In this view, the degree of synthetic activity and autolysis as well as the energetics of the wall are under some degree of physiological control, but what happens to any portion of the wall depends on the stress applied to the fabric and follows engineering rules similar to those that apply to man-made fabrics. These rules impose architectural constraints on the fabric, giving partial order to the wall of procaryotes, depending on both the types of cell morphology and cell cycle dynamics. This view of a less structured cell envelope models the wall of bacteria as a nonwoven fabric. Because the stresses are asymmetric in the wall surface, partial order develops. I will refer to partially ordered wall due to such physical force as a "carded," nonwoven fabric (49).

MAGNITUDE OF STRESSES SUPPORTED BY THE BACTERIAL WALL STRUCTURE

The surface stresses in a hemispherical pole are the same in all directions and are mathematically expressed by $Pr/2$, where P is the turgor pressure and r is the radius of the cell (23, 90). For the cylindrical part of the cell, the meridional (axial) stress is equal to $Pr/2$ as in the pole, but the hoop (circumferential) stress is twice as great (Fig. 1). The stress in the unsplit septum of a gram-positive organism should be zero as long as the surrounding external wall bears the stress generated by the hydrostatic pressure. However, there is stress on the developing constriction site in the gram-negative and the partially split portions of the pole developing from the septum of the gram-positive organism. Models for bacteria of other shapes can be similarly derived from the methods which engineers have developed to evaluate the stress in the surface of pressure vessels (23, 90) (Table 1).

In quantitative terms, the chemical effects of these tensile stresses must be highly important. I have calculated (43) that

TABLE 1. Stresses in portions of a pressurized cell with axial symmetry[a]

Portion of cell	N_ϕ, meridional or axial stress	N_θ, hoop or circumferential stress
Sidewalls		
Cylinder	$Pr/2$	Pr
Arbitrary	$Pr_2/2$	$Pr_2(2r_1 - r)/2r_1$
Poles		
Hemisphere	$Pr/2$	$Pr/2$
Ellipsoid	$\frac{Pa^2}{2(a^2\sin^2\gamma + b^2\cos^2\gamma)^{0.5}}$	$\frac{Pa^2(b^2-(a^2 - b^2)\sin^2\gamma)}{2b^2(a^2\sin^2\gamma + b^2\cos^2\gamma)^{0.5}}$

[a] Formulas taken from references 23 and 90. P = turgor pressure; r = radius of rod or hemisphere; r_1 = radius of curvature in meridional direction; r_2 = radius of curvature in hoop direction; a and b = semimajor and minor axes; and γ = azimuth angle.

the force on every bond in a hypothetical spherical cell whose radius is 0.8 μm and which is composed of a perfectly regular cross-linked monolayer of murein with 5 atm (506.45 kPa) of hydrostatic pressure is 5.85×10^{-5} dynes. While these choices of design characteristics for the wall are only rough approximations of the realistic in vivo situations, the forces probably are of this magnitude for both gram-negative and gram-positive organisms, but vary a great deal depending on the details of global and local geometry.

The effects of tensile forces in the wall of cells under hydrostatic pressure are twofold. First, any bond that is made and broken spontaneously even at moderate rates is more likely to remain open under tension. Such processes lead to the breakage of original bonds and intermediate formation of new weak bonds with different partners leading to the processes of "creep" and relaxation. This occurs in other materials such as plant cell walls, spun threads, and nonvulcanized rubber.

The second effect concerns covalent bonds. If the bond had to be stressed as an integral part of wall formation, the energy of formation would be greatly increased. Since work is mathematically related to the distance over which the force acts times the force, there is an additional bioenergetic cost to form a bond that is stress bearing at the time of formation. That is, unless the biochemical process utilizes extra "high-energy phosphate bonds," or their equivalent, net formation of stressed polymer may not be possible. If the distance that the enzyme has to pull the two reactants together is of the order of 0.1 nm, it would take at least one "squiggle" more if the bond were to form in the wall of the hypothetical cell with a monolayer of peptidoglycan (mentioned above) so that it was stressed as formed than if it were to form in a stress-free situation. The turgor pressure in this hypothetical situation would produce a stress of 5.85×10^{-5} dynes in peptide or glycan chains. This is equivalent to 17.6 kJ/mol (the typical high-energy phosphate bond is about 32 kJ/mol). It is for this extra energy requirement that the surface stress theory postulates that the cross-linking bonds to the existing wall are formed and then stressed (55).

This energy also lowers the energy of activation for hydrolysis by the same amount. While the stress in the procaryotic cell lowers the energy of activation by a small fraction of the energy of a typical covalent bond (typically 100 kJ/mol) (43), this is sufficient to greatly accelerate chemical, or enzymatic, action by as much as 10^5- to 10^6-fold.

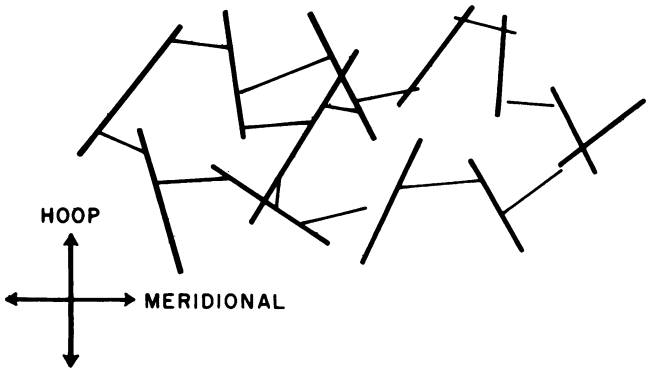

FIG. 2. Partial order resulting from growth and enlargement of the gram-negative sacculus. Because of the partially ordered nature of this basically two-dimensional structure, it is categorized as a carded, nonwoven fabric. This type of fabric is basically similar to the structure of a piece of paper or polyacrylamide gel. In the diagram, heavy and light lines represent, schematically, the glycan and peptide chains, respectively. A preponderance of the glycan chains are ordered circumferentially to the axis of the cell (i.e., in the hoop direction). This kind of partial order has been observed experimentally (109, 110). The predominance of fibrils ordered in the hoop direction, it is argued in the text, results from asymmetric stresses present during growth and formation of the wall. Those peptide chains that happened to be oriented in the hoop direction continue to be preferentially hydrolyzed by autolysins.

Because the stresses are twofold larger in the hoop dimension than in the axial dimension of cylindrical walls, sensitive bonds oriented in the circumferential direction should be more frequently cut than bonds oriented in the meridional direction (Fig. 2). This accounts for the finding that the cell walls appear to have a partial degree of orientation visualized in the electron microscope (108) owing to more rapid disruption of the wall with ultrasound and enzymes in the direction parallel to the axis of the cell.

DOES FORM AT THE BUILDING BLOCK LEVEL LEAD TO FUNCTION AT THE SUPERMACROMOLECULAR LEVEL?

Microorganisms pass through a cyclical series of morphologies that are characteristic of their growth and division processes; this means that they must construct in very special ways the peptidoglycan layer which defines their shape and contains them. Man-made buildings may be an appropriate analog to the form of bacteria. Given that buildings have complex and characteristic shapes, one could suppose that the structure of the building blocks might give a building its form. Alternatively, one could suppose that many differently shaped buildings could be made out of bricks of a single shape. In architecture as well as in biology, both statements are partially correct.

To make this difference clear, consider a biological example. The form of viruses is determined, in many cases, solely by the primary structure of the capsid protein (39, 70). Thus, tobacco mosaic virus codes for a protein that assumes secondary and tertiary structures that allow it to self-assemble into a helix with a particular pitch and diameter. But, the total length of the helical assembly of proteins does not depend on the properties of the smaller building blocks. The length of the helix of protein subunits does depend under some, but not all, physicochemical conditions on the length of the enclosed ribonucleic acid. In certain cases, disposable scaffolding proteins are used as jigs to determine how many subunits are permitted to self-assemble (37) into the virus capsid.

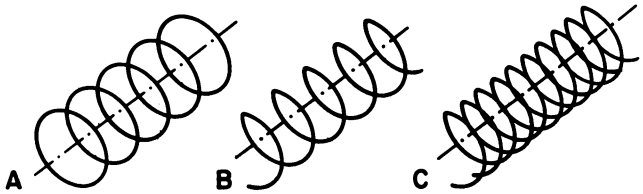

FIG. 3. Candidates of a regular type of fabrics for the structure of cell walls. The cross-links are not shown. Three regular structures are shown: (A) series of hoops of the Weidel and Pelzer sacculus (84, 113); (B) single-stranded helix of the Mendelson helix clock (76); (C) double-stranded helix model of Burman and Park (13).

When such a building block model is applied to bacterial morphogenesis, it could be imagined at one extreme position that the varieties of peptidoglycan fragments observed by hydrolysis and high-pressure liquid chromatography (25) have shape-determinative properties. Thus, the peptidoglycan is not like concrete, which can achieve any shape depending on the form into which it is cast, but has special pieces for special functions as in a jigsaw puzzle. Are there specially shaped "bricks" in the peptidoglycan that only fit together in a certain way? Clearly the final cross-linked character of the glycan determines the shape of the sacculus and allows the cell to contain and resist high osmotic pressure. At the other extreme, it could be imagined that any of the shapes of bacteria could be made with any of the variant structures of the disaccharide pentapeptide structures found in any of the procaryotes (92). If this is true, is there something like a template, or scaffold (37), or is the shape determined in some other manner?

How a Regular Wall Structure Could Be Made

The simplest imaginable way a rod of constant diameter could elongate is if its wall consisted of covalently closed rings of glycan encircling the rod. This is an extreme extension of the concept of Weidel and Pelzer (84, 113) (Fig. 3A). Following their ideas, I tried to imagine how this might be achieved from a mechanistic and energetic point of view. The perimeter of cells grown in minimal medium would be 2,500 muropeptide disaccharides long for *Bacillus subtilis* (35) as well as for *Escherichia coli* (13) at a moderate growth rate.

The saccharide chain would have to be covalently closed. The energetics of formation of this terminal linkages would have to be different from that of the other glycoside bonds. In principle, there is adequate free energy from the phosphate linkage for this process, but the geometry is wrong. Closure at the end of the "rib" around the cell could be created by one of the two types of transglycosylase activities present in the cell, namely, the one that splits the carbohydrate chain, but saves the energy in the form of an anhydro-1,6 linkage. The energy saved in the dehydration to form the anhydro compound can later be used to form a normal glycoside bond. This reversible process could, in two steps, create a covalently closed circular glycan molecule. It would, of course, necessarily create new ends that would require either elimination by turnover or incorporation in a non-stress-bearing part of the wall.

These glycan rings would bear the hoop stress and the peptide chains would bear the meridional stress from pole to

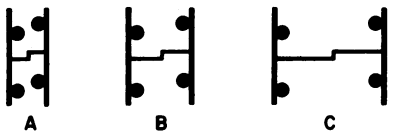

FIG. 4. Energetics of peptide cross-bridge formation and dissolution. Cross-bridge formation depends on the juxtaposition of the donor, acceptor, and transpeptidase. If the two glycan chains are held at various distances by the remainder of the wall structure, the peptide chains can only be linked when they are in a particular conformation. Since the stress-free conformation of the tetrapeptide is compact (2), formation of cross-bridges requiring an extended conformation will happen more slowly in example C than in example A or require an additional (external) source of free energy. Conversely, if other factors are equal, the negative of the free energy of hydrolysis is greater for larger interchain separation. Consequently, a stress that increases the interchain displacement will cause the energy of activation for hydrolysis to decrease and the equilibrium to be further in favor of hydrolysis. See text and reference 43.

pole. These rings could serve as templates for the insertion of nascent rings in one-to-one alignment with existing rings. Two new sets of peptide linkages would form, and one set of stress-bearing ones would have to be cleaved. If this procedure were followed exactly, it would cause elongation without allowing diameter to change. But turgor pressure and the resultant tension leads to a tendency to skip and to form gaps, allowing the cell to become wider and rounder; consequently, these would have to be resisted by the forces involved in the templated pairings. The templating process being hypothesized would involve a counting or a one-to-one pairing of the saccharide residues on the two adjoining stress-bearing strands with those on the nascent strand. It is essential that this process link only a donor peptide from the nascent strand with the correct frame to the correct acceptor peptide on the correct stress-bearing strand. Incorrect peptidoglycan linkages could not be permitted to form since the process must make a perfectly regular product and such events would create nongenetic, but heritable, mutations. Molecular models show that the peptides are long and flexible enough that very special control would be necessary to maintain this precision pairing. This would require that the transpeptidase act as a Maxwell's demon (103). Information theory teaches that information is the equivalent of negative entropy; consequently, an enzyme that must choose between chemically equivalent reactants must have available more free energy than one that does not make such distinctions.

Extra thermodynamic work would also be required to limit peptide bond formation to the pair of currently joined stress-bearing strands since the peptide chains could be extended by thermal motions of pentapeptide donor and a recipient tetrapeptide to nonadjacent strands. Therefore, energy would be needed beyond that inherent in the D-Ala-D-Ala bond in the meridional direction as well (Fig. 4). Because the transpeptidation reaction is essentially a zero free-energy process, cross bridge formation only proceeds because the free D-alanine concentration remains low. There is no known additional potential source of negative free energy available outside the cytoplasmic membrane. These are strong liabilities of my elaboration of the strict interpretation of the Weidel and Pelzer model and cause me to reject a regular structure and mode of formation of the wall murein.

Other Reasons for Excluding a Regular Structure

While the thermodynamic reasons are sufficient to exclude the "constant perimeter-belt" model, there are diverse experimental observations excluding models of this class: (i) the glycan chains are only long enough to reach a small percentage of the distance around the cell (92); (ii) the peptidoglycan is not a chemically regular structure (25, 33); (iii) not all possible cross bridges are formed (92) at any particular time as required by a template process because they would need to be counted even if not linked; and (iv) some cross bridges may form between peptides attached to muramic residues residing on the same chain or on nearby but not adjacent chains. From the structural point of view, the former pairings would not help bear the surface stress and would prevent stress-bearing bonds from forming. Finally, cross-links to nonadjacent chains would create imperfections that would behave as inheritable "mutations" during subsequent cell cycles.

Studies on peptidoglycan structure by X ray, model building, and computer energy minimization have been numerous (1, 2, 5, 6, 11, 12, 24, 64, 65, 81, 111). They provide evidence of order, but only over distances of the size of a disaccharide-peptide unit. There is essentially no order over a larger scale. It is to be noted that all experimental X-ray studies had to be done with specimens in the relaxed state. It is known that, with such necessarily randomly cross-linked substances as rubber, higher order appears in the diffraction pattern when the sample is stretched to near its breaking point (103). The computer studies (64, 65) to minimize the energy are for nonmechanically stressed molecules; the work (primarily from Labischinski's laboratory [64, 65]) demonstrates that the oligopeptidoglycan would (i) be fairly linear in the glycan dimension, (ii) have the peptides radiate from this chain in a helical arrangement, and (iii) have the peptide chain folded into a very compact mass very closely in apposition to the glycan chain. Of course, the second and third rules should not hold when the structure is part of a fabric, particularly if it is stress bearing.

Before abandoning all regular models, let us proceed further with the template notion as a logical exercise. Assume that, when formed and as they exist in the growing organism, all glycan rings appear as covalently closed circles and are cross-linked at regular intervals of their structure to both adjacent rings. One would then need to explain the observed shortness of the chains, incompleteness of the cross-linking, and complexity of the fragments resulting from lysozyme digestion as artifacts of the biochemical and analytical procedures. Such ad hoc explanations are improbable, not impossible. Continuing to argue for the insertion hoop model, possibly the extra free energy could be provided if the peptide originally contained a third D-alanine residue or if two pentapeptide chains were linked with the loss of both of the second D-alanine residues during the transpeptidation event. Again, this is improbable since the transpeptidase seems to execute a simple reaction, but it is not impossible since the synthase class of enzymes do equivalent chemistry.

Of course, two other problems posed by gram-negative rod physiology would still have to be solved to have an acceptable replication hoop model: a special mechanism to permit the diameter of the gram-negative rod to change under different growth conditions and a special mechanism to cause constriction and division.

How a Wall of Irregular or Random Structure Could Be Made

Biosynthesis of the sacculus is much easier to understand if its structure is irregular, because then the polymerization

can be assumed to be basically a random, collision-controlled reaction. Oligosaccharide chains of variable and uncontrolled numbers of disaccharides would be secreted. Their average composition and chain length distribution would depend on the species and on other circumstances. Then they would be polymerized largely to existing murein by membrane-bound transpeptidases and transglycosylases, again as the opportunity presented itself. Because the peptide chains normally form into a secondary structure with compact form, the cross-linking of nascent chains to chains not immediately adjacent to the peptides on the older wall is restricted, but not eliminated. The donor and acceptor peptides, even without the additional linking peptide portion present in some species, are certainly capable of ranging over a radius of 2 to 4 nm from their attachment to the muramic acid residue on the glycan chain. Consequently, a mat or feltlike structure would be formed with little or, in some cases, no geometric order among the fibers.

Some order may develop because the existing wall is under asymmetric stresses (see below). The wall growth as noted before, would depend on the turgor pressure, radius, localization of synthetic and autolytic activities, and energetics of synthesis and autolysis. For cylindrical cells, elongation depends on the existence of relatively rigid poles to support the sidewall growth (42–44).

Because the secretion of wall material can only take place directly adjacent to the cytoplasmic membrane, it might be anticipated that the wall would become, by chance, thicker in some regions of the sidewall than in others. However, this would self-correct for the gram-negative organism because then the stress would be shared between layers and, as indicated above, reduce the rate of autolysis, which may account for the extra thickness observed in recent ultrastructure studies (32). For gram-positive organisms, an accidental extra thickening would make the outermost layer sufficiently peripheral to favor its autolysis and sloughing (38, 47, 53).

From the perspective of many microbiologists, the major objection to the surface stress theory is that it is not clear how such a mechanism could function to give a stress-resistant layer capable of enlarging, dividing, and separating. However, mathematical and biophysical analyses have shown how this could happen in the absence of proteins capable of doing mechanical work (42–46, 50, 52, 55). Actually, the major difficulty with our general model is that it does not explain the growth strategy of all procaryotes. At present, biophysics is incapable of explaining the shapes of spirilla and corynebacteria. Spirochetes represent a special circumstance because of their more complex structure and are not covered by the theory. It also cannot explain the shape of the square bacterium described by Walsby (112), but it need not because this organism is anomalous in having no turgor pressure. A major failure is that it cannot account for the shape of *Spirilloplasma* spp. It will be seen below that multicellular helices of organisms really do not pose a significant problem to the surface stress theory. For simple rods and cocci, the theory has met with success in predicting detailed shapes and alteration in those shapes under different physiological conditions.

Another difficulty, or strength, depending on point of view, with the surface stress theory concerns autolysin action. As Weidel and Pelzer (113) pointed out, hydrolysis of stress-bearing bonds is necessary for wall enlargement. The problem is that an autolytic event in the stress-bearing wall creates an imperfection and causes a redistribution of the load. This may cause the stress in some adjacent bond to become much higher, which would favor failure of that bond (with or without the mediation of an enzyme). This process, as first pointed out by Griffith 70 years ago (28), leads to the formation of cracks and fissures (the domino theory). This crack propagation process is well established in material sciences and geophysics.

Consequently, the action of autolysins must be carefully controlled to function properly in permitting enlargement without catastrophe. Some alternatives for this control have been proposed for the cell wall turnover of the gram-positive rod (56), but little can be said for the gram-negative rod except that the relevant enzyme(s) (presumably endopeptidase[s]) must be able to classify bonds in ways beyond the abilities of most enzymes. Such a "smart" enzyme must cleave a sensitive stress-bearing bond only if the structural integrity of the wall will not be breached. This subject is discussed further below.

RISE AND FALL OF THE CONCEPT OF A WALL GROWTH ZONE

A number of models have been proposed in which the peptidoglycan structure is assumed to grow at a limited number of sites in a localized portion of the cell wall. While the replicon model of Jacob et al. (34) required that wall be added in a particular region of the cell, there was no implication of a systematic and regular addition of murein moieties. The implication was only that it occurred between the attachment sites for the chromosomal origins. The simplest model to explain segregation is that zones exist which foster wall growth on only the central side of the chromosomal attachment site, and therefore none at all takes place on the polar side.

As a primitive mitotic apparatus for the primitive procaryote, the original replicon model has many strong points in its favor, but it also has difficulties. We have suggested a modification of the original replicon model (the "renovated replicon model" [58]) in which the origin and terminus regions of the chromosome are attached to the junctions of the pole and the sidewalls (or directly to the apex of the poles). A key point in this model is that it does not require zonal growth, but instead is consistent with insertion of murein over the entire cell. Insertion takes place without a requirement for a localized growth region or a regular mode of insertion, though such localization is not prohibited. A second key point is that our modification allows the cell to accurately position the next cell division site. As far as I am aware, this is the only model that can account for the observed precision of the mechanism dividing the cell in half (but see the discussion of the helix clock below).

The popularity of the idea of a growth zone from the replicon theory led to the concept of a functional growth zone of fixed size, location, and duration of action. An additional reason for the acceptance of the zonal concept is the unassailable evidence that the growth of *Streptococcus pyogenes* occurs zonally (17). At growth zone sites the wall could be imagined to enlarge at a constant rate because of some constraint due to the local nature of the mechanism. This would require a single region involving secretion and localized polymerization that adds new wall at a constant rate. This led to the development of ideas about linear growth expressed by Kubitschek (62, 63), Pritchard (89), two papers by Zaritzky and co-workers (93, 116), and papers from the laboratory of Karamata (96–98). The concept that wall growth is a regular process because the enzymatic machinery functions in a processive way is basically quite attractive. Moreover, this hypothesis could be tested experimentally.

One approach was autoradiography. Experiments performed with tritiated diaminopimelic acid, both before the replicon theory was proposed (107) and subsequently in Jacob's laboratory (68), demonstrated that wall enlargement of *E. coli* took place in a diffuse way all over the cell surface. Later studies, however, with autoradiography (94, 99) and the size distributions of cells taken from balanced growing cultures (116) provided experimental support for zonal growth of the cylindrical portion of *E. coli*. At about this time, my biophysical studies demonstrated that zonal growth of a cyclindrical structure is basically unstable (42, 43). Unless constructed as a regular structure of a woven fabric type or unless a cytoskeleton exists with the ability to control shape, such as eucaryotic cells possess, cylindrical growth will eventually lead to "blowouts." That is, growth is metastable and eventually leads to bulges that indefinitely and progressively get wider. I therefore reexamined the available autoradiographic data and concluded (43) that all of the earlier work is fully consistent with the notion that cylindrical extension in *Escherichia* and *Bacillus* spp. takes place by the diffuse addition of new material randomly all over the cylindrical surface. The pulse-label high-resolution autoradiographic experiments of Ryter and co-workers (94, 99) demonstrated that the intensity of incorporation on any region of the cylindrical surface is small compared with that in the region where cell division is about to occur. However, my reanalysis indicated that the low level of diffuse incorporation is adequate to account for the elongation process. I further concluded that it was not necessary to postulate, as had been done (31), that the wall insertion occurred first at a central site after which the material was (20) redistributed laterally to the cylindrical portion of the wall.

Thus, reanalysis of the earlier data together with the experiments of Verwer and Nanninga (109), the new experimental results of Burman et al. (14), and the technically excellent recent experiments from Woldringh's laboratory (114) are now all consistent with the diffuse insertion of oligopeptidoglycan moieties into the sidewall. Of course, the autoradiographic data cannot distinguish how many sites exist on the cell membrane for extrusion and cross-linking of oligopeptidoglycan. However, the autoradiographic results are inconsistent with the idea that the transpeptidase/transglycosylase molecules and the region where nascent oligopeptidoglycan strands are extruded in the cytoplasmic membrane are localized to a discrete, narrow, cylindrical zone. Consequently, the simplest interpretation is that the accretion events augmenting the cell wall are not systematic, but more or less random. This probably also applies to the constriction sites where poles of rod-shaped organisms are developing. The pace in these regions is faster. Any alternative hypothesis for the autoradiographic data would require the existence of a complex mechanism to control the spacing and functioning of multiple wall-synthesizing units.

CURRENT MODELS THAT ASSUME A PRECISE, REGULAR MECHANISM FOR MUREIN ADDITION

Old concepts die slowly; a regular mode of wall enlargement makes much simpler predictions and therefore is very attractive. Two groups have championed mechanisms of wall growth in which the oligosaccharide is spun through the cell membrane and systematically linked and inserted into regular helical structures. Their proposals are analyzed in the next two subsections.

Helix Clock Model of Mendelson

Mendelson's helix clock model is related to the multinet theory for the elongation of higher plant cells (89, 91). The latter will be described first even though it is no longer as popular with plant physiologists. In the multinet theory, a layer of continuous cellulose fibrils is assumed to be laid down as a tightly wound helix around the cell membrane (Fig. 4B and C). As the cell subsequently elongates, the pitch of a completed nongrowing helix gradually increases. At the same time, a new helical layer is laid down, tightly wound as it is formed, inside the old. Elongation in this pattern, with new helices that are formed like compressed springs and that subsequently become extended in length, continues as long as the outermost helix can still be distended further. This pattern seems to apply only to plant cells that are terminally differentiated, because this scheme requires that eventually growth must stop when the outermost helix becomes fully straightened. The preplanned expansion has been achieved. In principle, with an obligatory sloughing and turnover of the outermost layer of wall, this is a mechanism that could allow for indefinite growth and replication; thus, it could be consistent with an inside-to-outside growth pattern observed for gram-positive rods (53, 56, 58, 86, 87).

Mendelson's model for gram-positive bacteria (74–78, 105) results from studies of mutants of *B. subtilis* that grow into filaments because of deficiencies in the autolysin system. These filaments, as well as some other naturally occurring organisms and the wild-type strain under certain conditions (117; D. K. Mahnke, K. E. McManus, and A. L. Koch, unpublished data) can grow into a complex system of helical cables. Such systems can be drawn into macroscopic threads (105). Mendelson presumes that the helical nature of the macroscopic threads results from some underlying regularity in the peptidoglycan structure of the individual cells and led him to propose the helix clock model.

The helix clock model was reviewed in 1982 (77). The observed helices require fiber bundles with at least two chains of cells. Under most conditions, single linear chains have little tendency to assume a helical shape. The helix-producing interaction depends, at least in part, on the attractive interaction between the rough external surfaces of the chains of cells. Mendelson presumes that these surfaces have an inherent helical arrangement and that a second filament with the same arrangement, or a foldback of the same filament, will adhere and cause the formation of the twisted chain.

In the first paper on helical growth (74), he demonstrated that the outgrowth of spores of chain-forming organisms with a second mutation that left the distal cell attached to the spore coat developed into coils as if the cylindrical portion of the cell wall rotated as it was formed. I have proposed an explanation for the initiation of helical structures that does not depend on the regularity or helicity of the peptidoglycan on the cell surface (49). This explanation concerns Mendelson's original system or the initial interaction of a linear filament when it folds back on itself and self-adheres because of the positive attraction of the surface polymers for each other. In this case the looped portion is under compressive strain on the inside arc of curvature and tensile strain on the outer arc. These strains become resolved (as the result of Brownian motion) into torsions leading to the formation of a helix. In this way, the length of all original meridians is the same. Then the torsion becomes zero.

Mendelson's later work (75) showed that the outgrowth from spores was not required for helical growth. Rather,

FIG. 5. Early stages in the formation of aggregates of filaments of *B. subtilis*. Diagrammatic observations of Mahnke et al. (unpublished data). The predominant morphological forms at the times indicated after $\geq 10^4$-fold dilution in Luria broth of an overnight culture of wild-type strain 168 are shown. Even though these cells are wild type, at this high dilution they grow as filaments that initially appear to be linear with no helical character. The initial interaction appears to be by both chain-chain aggregation and by foldback. At a later stage than shown, the structures grow into the typical macrofiber such as described by Mendelson (75) for autolysin-deficient cells. These macrofibers are more tightly coiled than were the earlier aggregate stages, such as depicted here. The macrofibers of this autolysin-sufficient organism dissociate into motile, predominantly single cells when the culture density reaches 10^8 per ml.

growth from single cells or short filaments present in overnight cultures of autolysin-deficient strains also gave rise to helical bundles (the single cells used to start the cultures arise because the stains are not completely autolysin defective). This system is quite different and requires different explanations. In this case, the single cells grow into straight chains of cells that progressively become longer and very occasionally fragment. Depending on the dilution factor of the overnight culture and the genotype, the first interaction may result from either fold-back or side-to-side interaction of different filaments. In either case the structures initially show little regularity or helical character (Fig. 5). The hand of the helix may reverse, and the interaction is not tight. With further development, largely by fold-back of the multiple-stranded self-interacting structures, the larger structures become more regular and the helical coils become tighter.

An alternative explanation (49) for the forces that lead to these helix structures assumes that cells in the center of such bundles grow and elongate more slowly than cells on the periphery. The internal biomass should be limited for nutrients and oxygen. The latter would be extremely important for this strict aerobe. Because of the lateral attractive forces and the existing helical structure, severe tension would develop, tending to stretch cells in the middle and compress cells on the edge. In a growing plant meristem (apex or root tip), the cell-to-cell interaction is very strong so that there is no slippage in the cell-to-cell interactions; consequently, the forces are not dissipated and remain in the multicellular structure. For macrofibers, only weak bonds hold the aggregate in a side-by-side interaction, and there can be slippage and creep that will resolve the stresses by forming tighter helical arrangements in the more rapidly growing external elements than nearer the center (Fig. 6). This process would tend to regularize the helical arrangement because to unwind or reverse the pattern would require a good deal of additional energy. Thus, it is probable that the macrofibers result from torsion between filaments of cells in the bundle adhering to each other and centrally located cells growing at slower rates than surrounding cells. For example, the experimentally observed results of reference 74 then would arise if the cells in the middle of the cable grew threefold slower than the ones on the surface of a thick macrofiber containing 1,000 cell filaments.

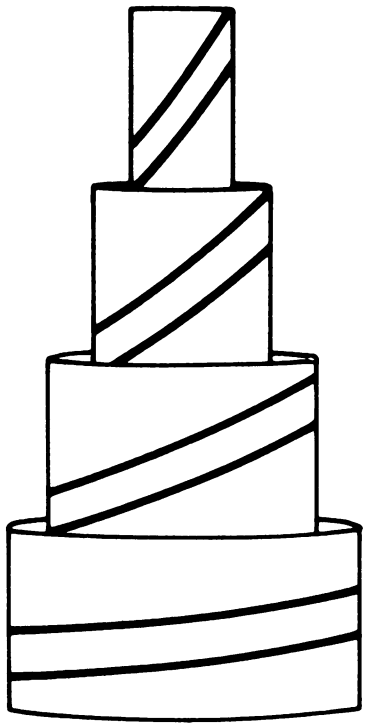

FIG. 6. Idealized growth of a bundle of filaments. A dissection through a bundle is shown diagrammatically. It shows the highly idealized structure that would result if a bundle were to form from filaments initially packed parallel to each other that then grew at different, slower rates depending on the distance from the nutrient source in the bathing medium. See text and reference 49.

An additional force leading to a helical structure is also observed in the formation of ropes and cables; the winding of helical structure leads to more contact between the surfaces of the strands. Since we do not know the forces that lead to the side-by-side attractive interaction of the cell chains, the quantitative aspect of the resultant forces cannot be appraised. Perhaps there are divalent cation bridges between the negative charges on the wall. Perhaps there are some hydrophobic interactions, but we (Mahnke et al., unpublished data) have been unable to demonstrate either. Perhaps the relevant groups extend some distance beyond the surface. Strands of material can be observed in the electron microscope when stained with cationized ferritin or ruthenium red. The farther that some loosely held polymers can extend from the compact portion of the wall, the more the helical folding would increase the surface contact and increase the interaction energy.

In principle, the mechanisms considered above for helix formation would result in 50% left-handed and 50% right-handed helices. Once started, growth would tend to reinforce any existing helical structure. But a random handedness is totally contradictory to Mendelson's experimental observations. Under many particular sets of conditions, all helices found in the population are of the same handedness. Moreover, the handedness can be influenced by genetic and nutritional conditions. In some cases, the helical nature can be changed by alterations in the environment. Especially critical is the observation that it takes one to two generations after the nutritional change before morphological changes commence. As yet, no direct evidence has been presented to support the suggestion that a regularity in the primary

structure of the murein is the cause of macrofiber formation. Other bacterial species naturally grow in similar arrangements. For example, helical structures, called cords, are formed by *Mycobacterium tuberculosis* (19).

Mendelson's studies are relevant to the present discussion because he has developed a "helix clock model" of the cell cycle (76). The idea is that the new growth of peptidoglycan is in the form of (polysaccharide) "strings," of a fixed length comparable to at least the circumference of the cell and possibly many times larger. The strings are imagined to be wound around the cell surface. He argues persuasively that a clock can be constructed if the first string added during a cell cycle is added in an almost circumferential manner and the successive strings are inserted (intercalated) adjacent to the earlier one and cause the helical strings formed earlier to be deformed at a lower pitch. (Note the difference in the multinet and the helix clock models: in the latter, the new strands are intercalated; in the former, they are laid underneath.) At the limit of cell wall growth, the pitch becomes essentially zero. In principle, if this final straightening event triggers a cellular process, it does allow the cell to define a duration for wall growth corresponding to a cell cycle and could control the timing of cell cycle, segregation of chromosomes, etc. The part concerning the strings, however, is only a portion of that needed for a functional clock mechanism. Evidently a great number of other special conditions must be fulfilled so that the system can function as a clock, and thus the mechanism functioning in this manner in total must be very sophisticated and complicated. The one feature we are concerned with in this review is that the strings must be made processively and therefore necessarily at only a few discrete sites. To modify the model to include a large number of sites would be very difficult. That would require that the actions of the multiple sites be precisely coordinated with each other.

It is instructive to calculate the speed with which a single assembly would need to function to form cell wall at a sufficient rate. The mean number of disaccharides in the wall of a *B. subtilis* cell during growth in rich medium is 6×10^7 (35). If this number is made during a doubling time of 20 min, and if one chain were forming at a time, it would have to elongate at the rate of 5,000 disaccharides per second. This is 60 times faster than the speed at which holopolymerase III elongates deoxyribonucleic acid (61) and seems large because an enzyme or enzyme complex must move systematically during function while bound to the membrane. There are thousands of the penicillin-binding protein molecules in a cell and perhaps 100 of the types suitable to be involved in transpeptidation (33). If, instead of 1, there were 100 processively elongating chains, then the speed need only be 50 disaccharide units per second. While this is a plausible rate of disaccharide addition, it would be untenable for the helix clock model to assume multiple sites, because, as noted, it is difficult to imagine a solution to the problem of the coordination of the individual action and extent of action of synthesis sites.

A further difficulty is that the helix clock model would also need additional features to explain how the three-dimensional thickness of the gram-positive wall would be achieved. The upwelling of the peptidoglycan in the gram-positive rod (86, 87) would seem to imply that the outside surface of the cell wall should grow as strings parallel to the cells' axis because they necessarily must be composed of old strands.

Double-Stranded Helix of Burman and Park

The other model proposed recently positing a regular peptidoglycan structure is that of Burman and Park (13). Their point of departure was their measurement of the time course of the incorporation of diaminopimelic acid into the peptide cross bridges of peptidoglycan. The secreted pentapeptide first serves as a donor, losing its terminal D-alanine in the transpeptidation process. Presumably the D-alanine is lost into the medium. Later, the cross bridge is hydrolyzed (endopeptidase?) and each half is capable of forming a new cross bridge. Of necessity, it must function as a recipient (an acceptor) in this secondary transpeptidation process. Burman and Park's interpretation of the detailed time course observed in pulse-chase experiments is that peptidoglycan is inserted in a special helical way into the existing cell wall cylinder. They found that in short pulses a significant fraction of the recovered label is in the form of acceptor. Consequently, they postulated that two new strands are inserted between an older pair of strands and both new strands are elongated processively. It seems obvious that, in order to serve the structural role, these helical strands as formed must run many times around the length of the cell though they may be subsequently cut (by autolysins and/or transglycosylase?). A theoretical objection to the Burman-Park model, as formulated, is that the pitch angle of the helix should progressively decrease (see previous section). Consequently, some additional provision of the published model is needed.

An alternative explanation of their data (diffuse model) is that there is random insertion of new oligopeptidoglycan into the old wall all over the cylindrical surface (see above). However, at sites of constriction where the local activity is very high (see above), incorporation is extremely rapid. At the sites of constriction, donor peptides become acceptor peptides very quickly. A simplistic model that averages the contributions in pole and sidewall to predict the kinetics of the ratio of radioactivity in acceptor molecules to that in donor molecules is as follows. Let C be the radioactivity incorporated into the side walls during the pulse, F be the fraction of the wall that is pole, T be the time, and μ be the growth rate constant. Then the radioactivity from a short pulse in the donor compartment of the sidewall will decay exponentially. Its radioactivity will fall as $Ce^{-2\mu T}$. The factor 2 appears in the exponential expressions because two bonds must be formed for every one cut during exponential growth. The fraction lost will reappear in the acceptor compartment whose radioactivity will increase as $C(1 - e^{-2\mu T})$. On the other hand, the radioactivity of the poles in both the donor and acceptor positions remains constant and equal to CF in this simplest model. The acceptor donor radioactive ratio (ADRR), taking into account the contribution of the sidewall and the poles, is given by: $\text{ADRR} = (1 - e^{-2\mu T} + F)/(e^{-2\mu T} + F)$. This function was used for a computer program to simulate a 10-min pulse. The results with $F = 0.25$ are shown in Fig. 7 superimposed on the experimental results of Burman and Park (13).

This diffuse model is simplistic because it assumes that during the pulse part of the experiment the ADRR of the cylinder is zero. Actually, it should be slightly higher because the pulse usually lasts 10 min and because the membrane-bound transpeptidase may act repetitively within a small neighborhood. Also, a more realistic model would replace the assumption that the ADRR of the pole is initially 1 with the assumption that it is a little less than 1 because some of the pole wall may not be newly incorporated. The

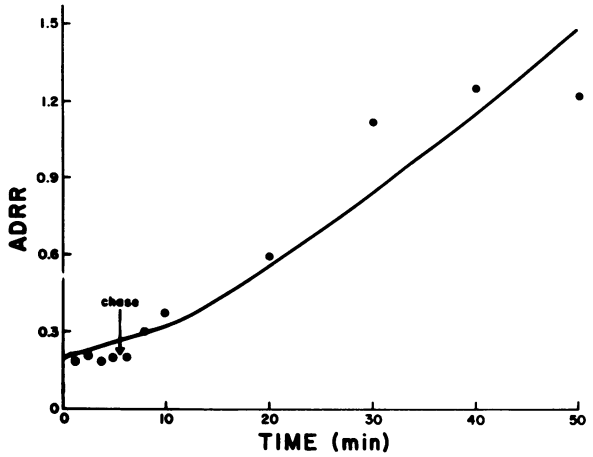

FIG. 7. ADRR during a pulse of diaminopimelic acid. A BASIC program was used to calculate the expected time course of the ratio of radioactivity from diaminopimelic acid incorporated into acceptor positions relative to that incorporated into donor positions. The program did so by superimposing the results calculated from the formula given in the text for a series of instantaneous pulses. In this way, the computer calculated the results for a case in which the experimental pulse lasted 10 min. It was assumed that a fraction, $F = 0.25$, of the cell wall was in poles. In this fraction the ADRR is always assumed to be 1. In the remainder, the ADRR is initially 0, but rises to infinity as the cylindrical wall is enlarged at random. The closed circles correspond to the data of Burman and Park (13) for this experimental situation.

presence of some previously old side wall material in the pole is suggested, because experimentally ADRR does not rise to the theoretical $(1 + F)/F$ or $1.25/0.25 = 5$ at very long times. Possibly, some material may remain in the donor form because it entered the cell wall at the poles which do not recycle peptidoglycan. Moreover, the chase may not be perfect, and therefore pool radioactivity may continue to bleed into the murein as donor peptides. Another possibility is that some cells may become quiescent, for any of a number of reasons, during or shortly after the pulse. This arrest in wall synthesis will lead to the maintenance of a low ADRR.

No Regular or Helical Structures for Cylindrical Wall

Both helix models for gram-positive and gram-negative rods require great sophistication on the part of the cell. Not only are a precise number of functional holoenzyme complexes needed, but also they must start at the appropriate time and place and elongate strands regularly and processively for a fixed time or until a fixed number of residues have been added. Otherwise, the functions required to determine the cell diameter or its length when deoxyribonucleic acid replication, termination, or cell constriction are to occur cannot be served.

In summary, a number of experimental facts and theoretical considerations are contrary to the concept of a regular structure for the production of the peptidoglycan of either gram-negative or gram-positive organisms. The partial order seen in electron microscope pictures appears to be real and can be understood in different ways for gram-negative and gram-positive rods (49). For the former, it results secondarily from the differential stresses present in the wall of a cylinder containing pressure. Growth leads to a partial order by asymmetrical autolysis. The data discussed in the previous two sections which had been taken in earlier studies as evidence for a helical nature of the peptidoglycan can be just as readily interpreted as arising in other ways. In the case of the helical filaments of *B. subtilis*, forces based on three different biophysical structures may function. For *E. coli* the two paired strands does not appear to be necessary when the effects of the special, high metabolic activities observed in constricting regions are taken into account. It should be noted that Park (personal communication) thinks that his experiments with nondividing cells exclude the poles as making a significant contribution to the ADRR at short times. However, it seems to me that all of the autoradiographic data are consistent in showing that there is a high degree of local incorporation in central constriction regions. Therefore, donors must become acceptors rapidly within the division sites, and a fraction of the wall label in a pulse will have an ADRR of essentially 1 because it represents the developing pole.

TABLE 2. Outline of fabric types

Woven
Knitted
Nonwoven
Fibers systematically ordered before cross-linking
Strong bonds only
Weak cross-links
Fibers randomly ordered before cross-linking
Strong bonds only[a]
Weak cross-links[b]
Certain weak links in both types of chains[c]

[a] This seems to be the case for enzyme-free walls.
[b] This is the in vivo situation for gram-negative cells when only the peptides are split by amidases and/or endopeptidases.
[c] This is the case for the gram-positive cells which contain both glucosaminidases and amidase. Note: The transpeptidase/transglycosylase penicillin-binding proteins presumably only act at the cytoplasmic membrane surface and not at the periphery.

NONWOVEN FABRICS, RUBBERS, AND GELS

Since there is no evidence for regularity in the wall structure, it is assumed in the rest of this review that the wall is a nonwoven fabric (29, 30, 69) composed of oligosaccharide chains of irregular lengths cross-linked via peptide cross bridges at irregular intervals to neighboring chains. There are two extreme types of nonwoven fabrics: (i) those in which all of the bonds are substantially the same strength; and (ii) those which include bonds that are much more fragile (Table 2). In the case of the peptidoglycan in bacteria, the covalent bonds in the peptides are comparable in strength to the weakest covalent bonds in the oligosaccharide chain. The hydrogen bonds (83) that can form within the wall can be important to its structure, but only in the absence of continuing stress. Consequently, neglecting this noncovalent aspect, the wall polymers in vitro are of the same type as vulcanized rubber, Lucite, or polyacrylamide (80, 101, 106).

The strength of covalent bonds is indeed great (83), if one considers the force that would be necessary to pull the chain apart in the dry state to form free radicals. For example, consider a spider web or monofilament of nylon. These materials are almost entirely held together with covalent bonds and on a weight-to-weight basis are many times stronger than steel. Silk on a weight-to-weight basis has 1.6 times the strength of hard steel (4). Imagine that a plastic was constructed by spinning oligosaccharide pentapeptide into a bath containing a soluble form of one of the penicillin-

binding proteins that functions as a transpeptidase. The resultant monofilament would have a strength greater than that of a nylon monofilament and many times that of silk or steel. If the glycan chains were well ordered due to the shear in passing through the orifice, the material would have little elasticity. If, on the other hand, the shear were small in passing the orifice, the product would be as strong, but now would be much more elastic. We could make a new type of "wonder" material by extruding an oriented sheet into the enzyme bath. Because the glycan chain would be oriented to a controllable degree, the resulting sheet could have little elasticity in one dimension, but have a two- to fourfold (5, 50, 81) capability for expansion (as does Spandex) in the other.

Some of the bonds in the murein are not as resistant to hydrolysis in aqueous solution as are others. Even so, they remain intact unless the pH is very much different from 7 and the temperature is much above the growth temperature of most bacteria. It should be noted that thermophiles, particularly those that live in acid waters, must have unusual cell walls. In fact, such hot acid environments are inhabited by *Thermoplasma* spp., which do not have a peptidoglycan stress-resistant covering (66).

It is not clear which enzymes serve the vital autolytic role during growth (although an endopeptidase cleaving the D-alanine–*meso*-diaminopimelic bond is the most logical candidate for *E. coli*). In any case, the autolysin cleavage events split only particular bonds in the peptide chain (33). As a result, the sacculus of the bacteria in vivo should have the properties of the second class of nonwoven fabrics with both weak and strong types of bonds mentioned above. The second class of nonwoven fabrics are akin to paper or thin slab gels of agarose or agar-agar because the cross bonds (usually hydrogen bonds or exchangeable salt linkages in these examples) are effectively weaker than the bonds within the chain.

The bacterial biopolymers actually belong to yet a further subdivision than shown above in the outline. They are nonwoven fabrics that are somewhat different from many of the man-made rubber or gels discussed above because of differences in their mode of formation (49). On the other hand, many other man-made products also have anisotropic properties created by shear and stresses at the time of polymerization. The cell walls are generated by polymerizing the nascent material to a structure which is already under stress and whose shape is the result of stresses on the wall in the past.

WALL FORMATION ACCORDING TO THE SURFACE STRESS THEORY

In this section, I summarize the results of our analysis of the mode of wall formation in a number of situations (Table 3). The gram-positive rod will be considered first, and the presentation is supplementary to the previous reviews (21, 54). The poles of gram-positive *B. subtilis* are made by the development of a thick septum under stress-free conditions (10, 51, 52). Then, by the "split-and-stretch" mechanism (10), cleavages bisect (56) the septum to produce the externalized pole wall. Although no more wall is added as the split septal wall becomes stressed, it stretches by 50% in area to form the final shape of the pole (51, 52). This is half of the increase needed if it were to expand to a hemispherical shape. After formation, the pole material turns over very slowly (53).

The thick sidewalls of *B. subtilis* are made quite differently than the poles by a process called "inside-to-outside" growth (53–56, 58). As mentioned above, this process involves the continuous addition of new layers underneath the existing wall (86, 87). The wall is deposited in an unstressed relaxed conformation and is probably composed of randomly oriented glycan chains. While this process goes on, the cell wall cylinder gets longer as the cell continues to grow. The result is that a lamina of the wall moves outward and becomes somewhat stretched. Still later, it becomes more fully stretched. As the wall is pushed to a still more peripheral location with growth, it is further elongated and undergoes some autolysis to permit continued expansion. Autolysin action is essential after elastic extension is no longer possible (53). Because there had been no orientation of the oligosaccharide during the polymerization, both glucosaminidase and amidase action is needed; this accounts for the presence of these two types of activity. Presumably, this is why gram-positive rods completely deficient in either or both autolysins have never been found. Growth of autolysin-deficient strains which have a low level of both enzymes leads to the accumulation of old murein fragments in the vicinity of cross walls in the filamentous habit of chain growth of these strains (43, 49, 96–98).

TABLE 3. Strategies for wall enlargement

Cells	Strategy	References
Cylinder		
Gram positive	Inside to outside	43, 46, 53, 55
Gram negative	Make before break	42–46, 50, 55
Poles		
Gram positive	Split and stretch	10, 38, 51, 52, 56
Gram negative	Variable T	44, 50, 55

According to the surface stress theory, the oligosaccharide chain of the gram-positive rod should be laid down nearly at random in the plane of the surface. However, the autolysin action should cause circumferential fissures in the peripheral aspects of the wall. This should be particularly evident in the case of autolysin-deficient organisms. Because the reasoning has been presented elsewhere (49), it will only be briefly discussed here. Basically, tension in the outermost intact layer will favor autolysis leading to longitudinal cracks along the cylinder. However, when residual fragments remain attached to a portion of the wall, part of the stress will be borne by them and thus the underlying material will be partially spared. This means that cracks and fissures will develop and then continue to deepen with further growth. Since for this part of the wall the stress is almost entirely axial, the old wall will tend to accumulate as rings or spirals. Just how many per cell and their relative mass will depend on the cross-linking within a lamina relative to that from lamina to lamina. Of course, the autolysin activity and the stress distribution in the wall or amount of interchain bonds will also be important in determining the morphological character of the outer aspect of the wall.

The stresses in the enlarging wall must lead to fractures in the outermost aspect of the gram-positive rod, giving it an irregular appearance (3) and a much larger external than internal surface (100). The shape and size of the pattern can be presumed to be as complex as that of the bark of trees since the same type of expansion forces are at work (although they are longitudinal in the procaryotic case and circumferential in the case of the tree). These patterns do not require a living entity; for example, drying mud exhibits cracks with similar patterns (104). Evidently the pattern in

these cases is dependent on the strength of the adhesion between material in the same plane and the adhesion between the planes with different expansion or contraction rates. Inspection of a number of species of trees makes it abundantly evident that a myriad of fracture patterns in the bark are possible.

With the thinner walls of the gram-negative rod, probably neither the split-and-stretch nor the inside-to-outside mechanism functions. Recently, it has been claimed by Hobot et al. (32) that the gram-negative wall is 14 nm thick, several times thicker than previously thought, and therefore might enlarge by an inside-to-outside mechanism (U. Schwarz, personal communication). While this is consistent with some estimates of the periplasmic space (102), it is contrary to many other observations. Such a gel structure seemingly could not exist in the living cell because of the hydrostatic pressure. Although very elegant new electron microscopic techniques were used by Hobot et al., I think that the wall fabric had become crinkled or undulated and it appeared thicker than it actually was. They utilized sections that were quite thick (50 nm) compared with the theoretical 1- to 2-nm wall thickness of a single layer of peptidoglycan. So I assume from our experiments (57) that, when the turgor pressure of the cell was lost, the fabric could be wrinkled enough to give the observed appearance. Woldringh and Weintjes (personal communication) have reanalyzed the available analytical data on surface area and murein content of *E. coli* and suggest that there are two layers of peptidoglycan in the growing cell. Our own survey (50) had led to the conclusion that the living cell was probably covered by a single partially extended, but complete, layer. I do not think that wall turnover at the level of removing previous stress-bearing elements occurs. The recent experiments of Goodell and Schwarz (26, 27) present evidence for peptidoglycan turnover, but no evidence for turnover at this level. I therefore discount the concept that the wall of the gram-negative organism is made by the inside-to-outside mechanism. It seems necessary that oligosaccharide must be bound before cleavages pull them into the stressed layer. This requires the wall to be thicker than one stress-bearing layer. The other constraint is that the penicillin-binding proteins are membrane bound and, even though some have their active sites for transpeptidation and transglycosylation on a flexible attachment to the membrane-fixed portion (B. G. Spratt, personal communication), permitting them to service a larger area and depth, it is not likely that linkages can be formed at any distance from the cytoplasmic membrane. Rather, insertion of oligosaccharide chains and cross-linking followed by cleavages of previously stress-bearing wall are necessary for both elongation and cell division.

An analysis of the biophysics of the situation has shown that constriction and pole formation could occur by a process, the variable-T mechanism (50), which is only a slight variant of that permitting cylindrical elongation (Fig. 8). For cylindrical elongation without scaffolding or template type of formation, it is necessary that $T = Pa$, where P is the turgor pressure, a is the radius of the cylinder, and T is the equivalent of surface tension. The latter is a measure of the energy requirement for surface enlargement of the covalent stress-bearing structure. To cause constriction, the process must be varied in some way to give more favorable energetics to the polymerization (50). It was shown that T must be halved locally to cause further growth to lead to constriction and division. Several alternative possibilities for how the cell might alter the energetics of a process that occurs outside the cell membrane have been listed (50).

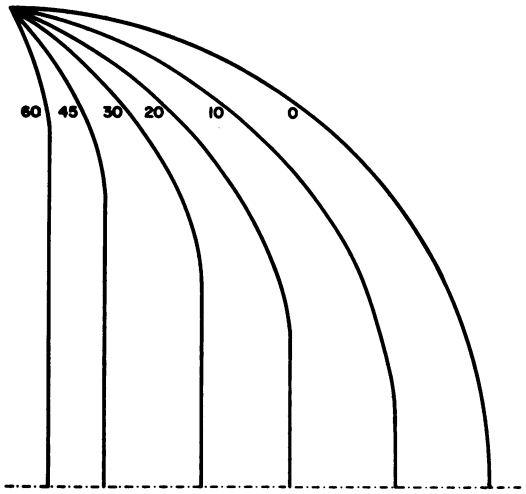

FIG. 8. Variable-T model for gram-negative pole formation. The tensile forces resulting from the turgor pressure causes the stress-bearing part of the wall to undergo cylindrical extension when three conditions are met. Under other conditions, constrictions may develop. The conditions for elongation are as follows: (i) the poles at the ends of the cylinders are of rigid diameter; (ii) the insertion of new material is diffuse over the entire cylinder (and not zonal); (iii) the turgor pressure, P, and radius, r, and effective surface tension, T, stand in the relationship, $P = T/r$. The quantity T is an energy term that is formally equivalent to surface tension considered in the physics of soap films. The variable-T model is based on the presumption that the energetics of external, stress-bearing wall enlargement is changed by the cell in the zone where constriction and ultimately cell division are to occur. The diagram shows the course of formation of a constriction site subsequent to a single change in the conditions for elongation. This is a discontinuous change of T by a factor of 2 from the value that leads to cylindrical elongation.

The simplest model for the growth of sidewall of a gram-negative rod is one in which oligoglycan chains are inserted through the membrane at random and cross-linked at random. Since the hoop stress is twice the axial stress (Fig. 1), the sensitive bonds oriented in the hoop direction will be preferentially cleaved. Assuming these bonds to be between a cross-bridged muramic acid and an alanine residue, two acceptor peptides result. To the extent that the bonds that form secondarily are in the axial direction, they will remain intact for a longer time than if they are circumferential. This selection, continued over time, would tend to align the peptide bonds in the axial direction and the glycan chains in the hoop direction. Again utilizing the terms of the textile engineer, the product is a carded, nonwoven fabric. A carding process in this vocabulary is a combing process that gives partial order to the fibers that are then to be spun or fused into a nonwoven cloth. Evidently, as further growth takes place or repair of defects in the wall occur, the fabric will tend to become disoriented. Countering this is the orienting effect of the preferential hydrolysis of the bonds situated in the hoop direction. The balance of these two processes would lead to a partially ordered state. In addition, it is also possible that the inserted chains will tend to be positioned by the structure of the extant wall so that the ligatures are formed in approximately parallel orientation to each other. Subsequently, the cylindrical wall of the gram-negative rod should be anisotropic in its elastic as well as structural properties. This is because the glycan chains are capable of much less extension compared with the nearly

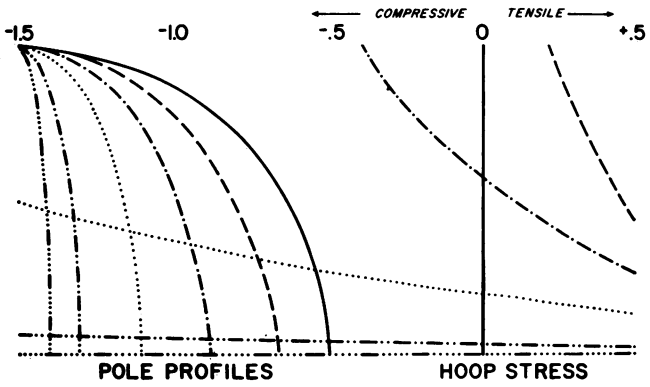

FIG. 9. Hoop stresses during formation of a division site in a gram-negative rod. Stresses have been calculated from a formula (Table 1) based on the approximation that the constrictions develop quickly to the cell axis and that the shape of each pole of the pair passes through a series of shapes that are ellipsoids of revolution.

fourfold increase possible for the cross-bridged peptide chains.

Turning to pole formation and shape, I will discuss the gram-negative case first. The constricting zone of *E. coli* is initially quite sharp with almost vertical walls, but eventually the pole becomes almost hemispherical (50) (Fig. 9). These shape changes can be understood in two equivalent ways. One is via the mathematics of surface films (e.g., soap bubbles) (44). Alternatively, they can be understood by utilizing the expressions for the hoop stress in shell structures given in Table 1. As the constriction zone is initiated, the hoop stress is compressive, and this forces the peptidoglycan to invaginate with further growth. However, as the constriction site evolves and proceeds inwards, the peripheral part comes to bear a tensile hoop stress. This pulls the pole outwards towards the hemispherical shape. Then the hoop stress is no longer compressive in any region.

This type of analysis can also be used to understand the formation of elongated poles of streptococci (Fig. 10). It was indicated above that the shape of *B. subtilis* is as if the septum were created as a stress-free solid disk and then split. Actually, the septum starts to split before it is completed. Apparently, it does not matter in this case whether the septum is completed or not, before the splitting begins. I

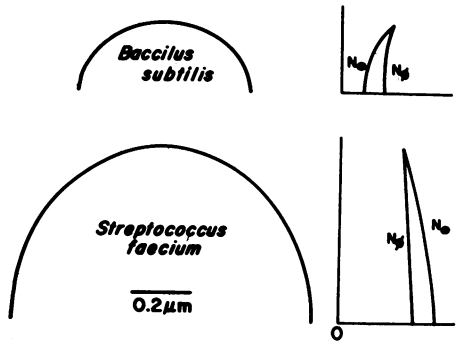

FIG. 10. Hoop stress during formation of an oblate and a prolate pole. Poles have been approximated as ellipsoids of revolution (Fig. 9 legend). (Top) Stresses for the shape typical of *B. subtilis*; (bottom) those for the shape of *S. faecium*. It can be seen that, in the former, the hoop stress is smaller near the axis, but the hoop stress is larger near the prolate pole of *S. faecium*.

infer that this is because the hoop stress at the site where additional wall is being laid down is small. On the contrary, in the case of the more pointed poles of *Streptococcus faecium*, the situation is quite different. The same analysis of stresses for the developing pole shows that the hoop stress is always tensile. This means that, after a layer of murein is added to the annular surface of the developing septum, it tends to be stretched before additional layers are added (A. L. Koch and M. L. Higgins, manuscript in preparation). Once this process is set into motion, it leads to a more pointed pole.

ASSESSMENT OF SACCULUS SURFACE AREA BY LIGHT-SCATTERING METHODS

Although the physical dimensions of sacculi of gram-negative organisms can be assessed with the electron microscope, using low-angle shadowing or other special techniques (5, 6, 115), the electron microscopic procedures have systematic errors because the cells are almost certainly distorted by all known methods of sample preparation. For this reason, we developed both a low-angle light-scattering apparatus and an appropriate theory to study the mean surface areas of sacculi under a variety of conditions (A. L. Koch and S. W. Woeste, manuscript submitted). With this technique the sacculi are examined in aqueous suspension in which they are hydrated and are not likely to be collapsed or wrinkled as are samples prepared for electron microscopy. Since this is a technique unfamiliar to most microbiologists, a nonmathematical explanation of the theoretical aspects seems appropriate and is given in the next section. A two-sentence summary is as follows. The area and other physical dimensions of the sacculus of gram-negative organisms can be found by comparing the intensity of light scattered over a range of very low angles with the predictions of the appropriate theory for the relative intensity of light scattered by populations of ellipsoidal shells of revolution. For sacculi from rapidly growing *E. coli*, the range of angles that are useful is 4° to 12°. The theory requires only one parameter to be fitted to the data. This is the radius of the rod-shaped cells. The surface area and contained volume are then readily computed.

The intensity of light scattered from a suspension depends on the mean dimensions of the sacculi, variation in those dimensions, eccentricity of shape, and distribution of orientations relative to the beam and detector. It also depends on a number of other factors (light intensity, concentration of particles, index of refraction of the particles, and distance from sample to detector), but these can all be eliminated by comparing measurements in which only the angle of observation (measured from the primary beam of light) is varied. The volume of the intersection of the light beam and the cone examined by the detector is another factor that varies with the angle of observation, but this factor can be eliminated with suitable calibration measurements. The only parameter having to do with the physics of the setup remaining in the mathematical formulation is the wavelength of light in the suspending medium. Of course, for laser light, this wavelength is very accurately known. The accuracy of the final calculated mean surface area of the sacculi is high because it is dependent on this single, accurately known parameter.

Low-angle light scattering can be used to estimate the size of growing bacteria (40, 41, 48), but the theoretical basis is more readily and fully justified for sacculi obtained from such cultures than for the cells themselves because the useful approximate physical model is precisely applicable.

For objects that are not small compared with the wavelength of light, the light rays from different parts of the object may reinforce or cancel each other in the beam of light scattered in a particular direction (40, 41, 48, 67). The calculation is easily possible if the retardation of the light going through the particles can be neglected. A calculation based on this presumption is called a Rayleigh-Gans approximation (36, 40, 67) and is suitable in this case. Since the sacculi are thin shells, the assumption is appropriate because a wave refracted from one part of a sacculus passes through other parts of the same sacculus for only a very short distance, the sacculus being so thin. Thus, the light is negligibly retarded and there is no correction needed for the phase of the light.

The procedure used depends on an appropriate mathematical function. These are called P functions: they allow the calculation of the intensity of light scattered relative to that scattered if the particle were compressed into an object small compared with the wavelength of light. Different formulas apply for different shapes of objects. To execute the program, the computer must be given three numbers: the axial ratio at birth of a cell; the ratio of the thickness of the wall to the radius of the cell; and the radius in the middle of the cell. The first two numbers are estimated from data available in the literature, but the values chosen are not particularly critical. The radius is the value of interest; it is found by a search procedure that minimizes the squared difference of the observed values and the predicted ones.

The mathematical expression for the P function (36, 40) needed in the numerical calculation for ellipsoidal shells of revolution has been derived by simplification of a formula for whole cells (18). The computer program developed for the data analysis of sacculi preparations is quite complex. It has to take the basic formula and compute averages over several parameters. One is to calculate the light-scattering functions averaged over a range of axial ratios for a fixed dimension of the semiminor axis to correspond to the change of shape during the cell cycle. For bacteria reproducing by binary fission, the algorithm presumes that this ratio varies from birth to division over a twofold range. The program also averages over all orientation angles of the asymmetric particles. The rest of the details are similar to the published program for light scattering by whole cells (48).

Mechanical Properties of the *E. coli* Sacculus In Vivo and In Vitro

Our resent studies (47) have shown that growing bacteria have a length 20% larger than the cell wall has when it relaxes following disruption of the cytoplasmic membrane with detergents. Filaments large enough to allow accurate length measurements were produced by growth in rich medium for 1 to 2 h after temperature shifts of mutants that form filaments at the nonpermissive temperature. The filaments were mounted on the glass surfaces of polylysine-coated rectangular capillaries in an apparatus allowing the bathing fluid to be replaced while the cells were viewed in the microscope. The degree of shrinkage was estimated by comparing the photomicrographs obtained before disruption of the membrane with ones taken after the phase contrast was lost. This loss signaled that the leakage had occurred and that the turgor pressure had been dissipated.

In a companion study (Woeste and Koch, unpublished data), the mean surface areas of pure sacculi preparations were estimated by the low-angle light-scattering method described in the previous section. For *E. coli* B/r H266, grown with aeration in rich medium at 37°C, the area of sacculi near the isoionic point (pH 4.6) and in 1 M KCl was 6.1 μm^2. Lowering the ionic strength to near zero increased the area to 7.8 μm^2. This was increased further at high pH to 13.2 μm^2 and to 20.1 μm^2 with the further addition of 10 M urea. It was raised to 24.5 μm^2 when the ionic strength and the pH were low in the presence of urea. Thus, the largest expansion above the most compact state that we were able to achieve was 302%. This factor of increase is consistent with a change of a nearly complete, compact, monolayer fabric without major open spaces on being expanded to the limit permitted by the most extended conformation of the peptide cross bridges (50). None of the suspension media should allow cleavage of covalent bonds, and the pH shifts were shown to be reversible with no hysteretic effects.

Comparing in vivo and in vitro studies reveals a significant difference. The natural degree of stretch in vivo is much less than the possible stretch in vitro. The in vivo sacculus is apparently expanded only 20% above the stress-free state. Compared with the 302% possible expansion of the in vitro sacculus, this suggests that the sacculus of *E. coli* is not stretched to its elastic limit in vivo.

Because the light-scattering method is new, it is appropriate that it should be justified and results be related to other, more familiar approaches. For this reason, the particular strain and culture conditions were chosen to be exactly as those studied extensively by Woldringh and collaborators in Amsterdam. It was found that the mean surface area of these organisms is 11.9 μm^2 (115). A similar estimate (10.9 μm^2) can be obtained from some of their other studies utilizing phase-contrast microscopy and by an agar filtration method followed by electron microscopy. The Amsterdam workers think that the cells shrink during the fixation procedure, but are expanded in the grid plane because the cylinders are flattened, and they estimate that the two effects compensate each other. Accepting this at face value, the area of the cell if it shrank when the phospholipid membrane was punctured would be 1.2- to 1.46-fold smaller. The former value applies if only the length shrinks, and the latter applies if the shrinkage of the sacculus is isotropic. The ranges of corrected values are in excellent agreement with the value of 7.8 μm^2 cited above.

Published studies of sacculi of the same strain under the same growth conditions, using the electron microscope, have reported surface areas of 12.1, 8.3, and 13.1 μm^2 (115). The difficulty with interpretation of these data is that the ionic strength at the instant that the sacculi were affixed to the grid is not known but can be presumed to be small in the agar filtration technique developed by Woldringh, in which high humidity is maintained during the procedure. In any case, these observations are not out of line with the other values.

The conclusion is that the growing sacculus of the gram-negative organism is stretched somewhat above its relaxed state, but is capable of a good deal more elastic expansion without recourse to autolytic enzyme activity.

PHYSICAL PROPERTIES OF GRAM-POSITIVE WALL

The key fact about gram-positive wall is that it is a thick three-dimensional matrix. There are variations in the chemical structure of the murein, or in the teichoic acid (or teichuronic acid), but basically it is a cross-linked elastic hydrophilic solid with both acidic and basic groups and internal hydrogen bonds.

Fundamental studies on isolated walls were carried out in the laboratories of Gerhardt (95) and Marquis (15, 16, 71–73,

82). By using various sized probe molecules, and measuring the excluded volume, the pore size was estimated. By choosing a large enough probe (high-molecular-weight dextran), the expansion and contraction of the wall could be studied as a function of pH and other environmental parameters.

The important result for our present purposes is that the fragments of walls of *Micrococcus lysodeikticus* swelled fourfold in volume from the most compact state of pH 4.3 and low ionic strength to the most expanded form found above pH 9 in the presence of 8 M urea (82). To relate this to our studies with *E. coli*, we need to estimate, from the change in the volume of wall fragments, the increase in area that the sacculus of this organism would exhibit. If the wall polymer is isotropic, the area would increase by the 2/3 power, or 2.52-fold. From the discussion given above, the wall should be far from isotropic because of its mode of formation and the effect of the anisotropic stress during cell growth. However, this is not too far from the elastic properties of the peptidoglycan supermacromolecule of the gram-negative sacculus, as discussed in the last section.

VULCANIZED RUBBER AS A MODEL FOR BACTERIAL WALL

Raw rubber consists of linear chains of isoprene units with a wide distribution of lengths, averaging about 5,000 units or 100 nm in length (106). In dilute solution, because there is free rotation about the single bonds, the chains have a random configuration. Vulcanized rubber has been randomly cross-linked with sulfur to produce a solid that has new properties. It can be reversibly stretched to 5 to 10 times its rest length and has a Young's modulus (at low degrees of strain) which is typically 10^5 smaller than for "hard solids" like metals or many plastics. These facts are consistent with the idea that in the relaxed state the portions of the rubber chains between cross-links are irregularly coiled in the state of greatest entropy. In this state the chain conformations can be described as approximating the paths that would result from a "random walk" process. The actual stress-strain curve at high degrees of elongation has a much larger Young's modulus, and at some point the rubber ruptures because it can extend no further without breaking covalent bonds. After one covalent bond breaks, the stress it bore is transferred to neighboring bonds, which are consequently easier to rupture. This leads to a "domino" type of, or Griffith, reaction, causing a tear or fissure and the eventual "failure" of the material.

Do the physical, chemical, and engineering studies on these materials cast light on the problem of the bacterial wall? I think they do. First, the theory developed to describe the properties of raw rubber provides a formula for the force that would develop in a single peptide cross-bridge chain of constrained length (106): $f = 3kTR/nl^2$, where k is the Boltzmann constant, T is the absolute temperature, R is the fixed distance to which the chain is constrained, n is the number of units in the chain, and l is the length of the unit that has free rotation. Whether the material were like a woven or a nonwoven cloth, this equation could be used to approximate the stress-strain relationships within small regions of the structure at low extension by multiplying the force, so calculated from the equation times the distance. We have found that the gram-negative sacculus in vivo shrinks by 17% when the turgor is relieved (57). Taking n to be 12 units and l to be 0.3 nm, the work required to decrease R by 17% from a distance of 2 nm between oligosaccharide chains is 4.9 kJ/mol or about 1 kcal/mol of cross-bridges. On the approximate assumption that the gram-negative wall is a regular monolayer with all peptides forming cross-links, the energy due to hydrostatic pressure within the living *E. coli* bacterium is 17.6 kJ/mol (43). Neither the 4.9 nor the 17.6 value is accurate. The former comes from an approximate formula; the latter will probably be revised downward with our new measurements of turgor pressure. However, the comparison suggests that the surface stress is mainly borne by entropic processes in the wall fabric.

WHAT THE MICROBIOLOGIST CAN LEARN FROM THE TEXTILE ENGINEER

To simulate the properties of a nonwoven fabric, Britton and colleagues have developed an elaborate computer program (7–9). The program constructs and then stretches a hypothetical fabric. This approach may be an important guide and provide a way to understand the mechanical properties of the cell wall. The program is outlined to emphasize the similarities, the differences, and the special problems with the microbiological situation. It operates as follows.

(i) The center point for fibers are chosen at random within a specified rectangular area. The fibers are assumed to be of uniform length, but their orientation within the plane of the fabric is selected by the computer at random. At each point corresponding to an overlap of two fibers, the computer selects whether a bond is formed and selects at random the strength of that bond.

(ii) In the next phase, the computer cuts out a subrectangle of smaller area chosen small enough that the distribution of fibers is not influenced by the size of the original rectangle. Thus, the sample chosen for the test is essentially the same as if the original rectangle had been infinitely large.

(iii) The dimensions of the test piece of fabric are selected to be 1 by 3. These proportions are chosen because that is the established policy of the American Society of Testing Materials (swatches 1 [2.54 cm] by 3 in.).

(iv) The computer then simulates the one-dimensional stretching process of the fabric. The computer does this in small stages. Each additional unit of strain causes deformation and changes in the angles of the fibers and leads to an increase in stress borne by the fabric swatch. When sufficient stress is applied to a bond, it breaks. Then the stresses become redistributed and become larger in some regions (and sometimes smaller in others).

(v) This process is repeated again and again until the fabric tears through.

This program builds in many of the properties of natural and man-made materials. However, it differs from what might be the case with bacterial walls. The principle differences are that, in bacterial walls, (i) the glycan chains are probably of variable length; (ii) the stress is in two dimensions (albeit asymmetrically) instead of one; (iii) the probability of bond breaking probably depend on the local stress and on the presence of an autolytic enzyme molecule; and (iv) as a very salient point, the fabric is one that grew and enlarged during a similar stretching and rupturing process. These points are being taken into consideration in developing a more realistic model of the procaryote wall.

CONCLUSIONS

This review has been far ranging in an attempt to bring the engineering done by humans for their own benefit into comparison with the engineering that living systems have

evolved for themselves. I have focused on the processes in the simplest sort of free-living organism, the procaryote. The arguments presented here are consistent with the belief that the procaryotes of today retained the simplicity of form and function of the earliest free-living organisms on earth. Then, it was necessary that the formation and function of the bacterial cell wall were achieved by quite simple means because there were neither contractile proteins nor supramolecular enzyme complexes that could do mechanical work. There appears to be no necessity to postulate a supramolecular machine that generates a regular structure by doing mechanical work and that is able to modify cell shape working against the turgor pressure. Rather, the procaryote is able to grow and divide by exploiting biochemical and biophysical principles. I can find no evidence that the procaryote creates an assembly line process that correctly aligns new synthesis of wall to precisely interdigitate with the old murein and generates cell morphology of a much larger scale than the disaccharide peptide. Actually, the surface stress theory delineates how the cell needs to function to control its wall growth and cell division in the absence of more elaborate mechanisms. This theory invokes only control for the loci within and upon the cell surface where formation and dissolution of the fabric occur. It implies that procaryote morphology depends on the peptidoglycan murein sacculus growth powered by the turgor pressure of the cell directed by the localization and function of the polymerizing and degradative enzymes.

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